Cell Stem Cell
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p53 Regulates Hematopoietic Stem Cell Quiescence

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DOI 10.1016/j.stem.2008.11.006

SUMMARY

The importance of the p53 protein in the cellular response to DNA damage is well known, but its function during steady-state hematopoiesis has not been established. We have defined a critical role of p53 in regulating hematopoietic stem cell quiescence, especially in promoting the enhanced quiescence seen in HSCs that lack the MEF/ELF4 transcription factor. Transcription profiling of HSCs isolated from wild-type and p53 null mice identified *Gfi-1* and *Necdin* as p53 target genes, and using lentiviral vectors to upregulate or knockdown the expression of these genes, we show their importance in regulating HSC quiescence. Establishing the role of p53 (and its target genes) in controlling the cell-cycle entry of HSCs may lead to therapeutic strategies capable of eliminating quiescent cancer (stem) cells.

INTRODUCTION

Hematopoietic stem cells (HSCs) can remain quiescent or they can enter the cell cycle and either self-renew or differentiate into multiple lineages. Although relatively quiescent, the HSC population must give rise to a hierarchy of differentiating progenitor cell populations that can replenish the blood system each day (Attar and Scadden, 2004). Blood cell production must also respond efficiently to hematologic stresses, such as blood loss, infection, or exposure to cytotoxic agents, via expansion of the HSC and/or progenitor cell populations (Passegué et al., 2005). These processes must occur without depleting the stem cell pool (Venezia et al., 2004; Forsberg et al., 2005).

HSC quiescence is likely controlled by both HSC-intrinsic mechanisms and bone marrow microenvironmental factors (Wilson and Trumpp, 2006). Several transcription factors have been shown to play key roles in HSC-fate decisions. Gfi-1 has been shown to restrict HSC proliferation and preserve HSC functional integrity (Hock et al., 2004; Zeng et al., 2004). HOXB4 and GATA-2 regulate HSC self-renewal (Krosl et al., 2003; Ling et al., 2004), whereas the Ets transcription factor MEF/ELF4 regulates both HSC self-renewal and quiescence (Lacorazza et al., 2006). Mef null mice exhibit greater numbers of HSCs (i.e., LSK cells), and Mef null LSK cells are more quiescent than normal. Early studies suggested a role for p21 in restricting HSC entry into the cell cycle and regulating HSC pool size and HSC exhaustion under stress (Cheng et al., 2000). However, the regulation of HSC self-renewal by p21 under steady-state conditions may be minimal (van Os et al., 2007). Several studies have implicated both the Ang-1/Tie2 and the thrombopoietin/MPL signaling pathways in maintaining HSC quiescence (Arai et al., 2004; Yoshihara et al., 2007; Qian et al., 2007).

The p53 tumor suppressor gene may regulate various aspects of hematopoietic cell behavior (Wlodarski et al., 1998; TeKippe et al., 2003; Chen et al., 2008; Akala et al., 2008). Although hematopoiesis in p53 knockout mice appears to proceed normally, numerous studies have identified roles for p53 in the proliferation, differentiation, apoptosis, and aging of hematopoietic cells (Shounan et al., 1996; Shaulsky et al., 1991; Kastan et al., 1991; Lotem and Sachs, 1993; Guzman et al., 2002; Park et al., 2003; Dumble et al., 2007). Moreover, p53 deletions and mutations have been found at high frequency in blast crisis chronic myelogenous leukemia and with some frequency in acute leukemia (Prokocimer and Rotter, 1994). In response to DNA damage, p53 can either elicit cell-cycle arrest or apoptosis, the typical outcome for mature hematopoietic cells (Wu et al., 2005). But p53 has recently been shown to negatively regulate neural stem cell proliferation and self-renewal (Meletis et al., 2006), and given that long-term reconstituting HSCs (LT-HSC) express high levels of p53 transcripts (Forsberg et al., 2005 and our data, see below), we examined the function of p53 during steady-state hematopoiesis. We find an important interdependency between MEF/ELF4 and p53 on HSC quiescence and identify two p53 target genes, *Gfi-1* and *Necdin*, that regulate quiescence in wild-type (and Mef null) HSCs. Our findings identify distinct roles for p53 in resting versus cycling cells.

RESULTS

Maintaining HSC Quiescence by p53

We have recently found that Mef/Elf4 null mouse embryonic fibroblasts (mefs) accumulate p53 protein and undergo premature senescence, which appears to be due to the ability of Mef to directly upregulate Mdm2 expression (G.S., Y.L., S.E.E., Y.M., K. Ohyashiki, S.M., S.D.N., unpublished data). We hypothesized that p53 could play a role in the enhanced stem cell quiescence (or the increased HSC frequency) seen in Mef null mice, so we examined p53 mRNA expression in primitive $Lin^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}$ (LSK) cells and in various myeloid progenitor cells (CMP, GMP, and MEP). p53 is most highly expressed in LSK cells (Figure S1 available online), suggesting that it could play an important role in HSC physiology.

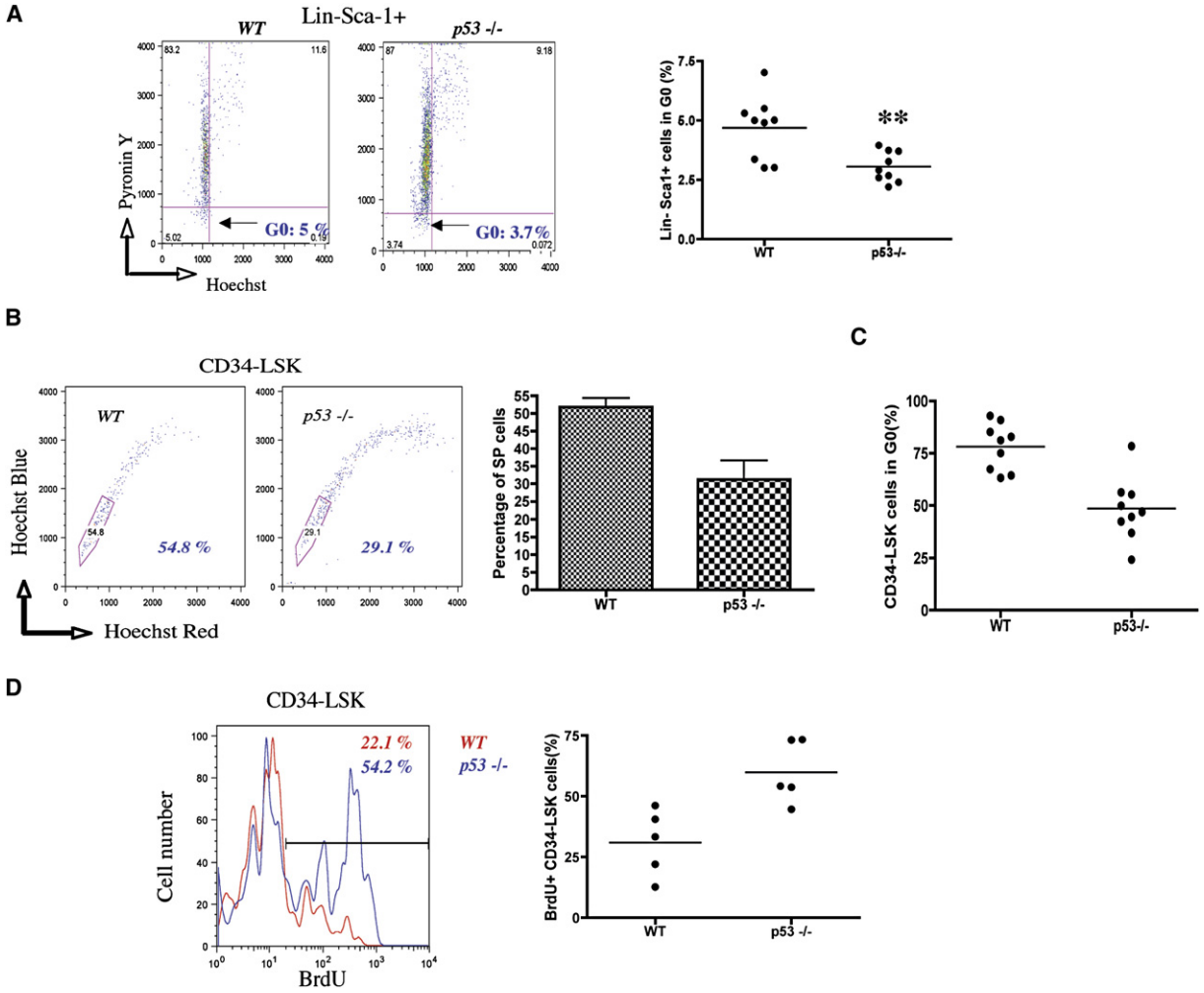

Figure 1. Maintenance of HSC Quiescence by p53

(A) Multicolor flow cytometry was used to determine the percentage of hematopoietic stem/progenitor cells ($Lin^-Sca\text{-}1^+$) in the G0 phase of the cell cycle (defined as cells with low Pyronin Y content that contain 2 n DNA [G0/G1]). Total bone marrow cells from wild-type and $p53^{-/-}$ mice were stained with Pyronin Y and Hoechst 33342. One representative experiment is shown on the left. The graph on the right indicates the mean percentage (±SD) of G0 cells present ($p < 0.005$, $n = 9$).

(B) Side population (SP) cells ($CD34^-$LSKs) from wild-type and $p53^{-/-}$ mice were identified by Hoechst 33342 staining and the use of blue and red filters. The bar graph on the right indicates the mean percentage (±SD) of SP cells present ($p < 0.003$, $n = 7$).

(C) Cell-cycle analysis of $CD34^-$LSK cells was performed by staining with Hoechst 33342 and Ki67 and analyzed by FACS. Data shown are the mean values ± SD ($p < 0.0001$, $n = 9$).

(D) The proliferation of $CD34^-$LSK cells was measured by in vivo BrdU incorporation over 48 hr. Greater proliferation of $p53^{-/-}$ $CD34^-$LSK cells was seen (60% versus 30% for wild-type $CD34^-$LSK cells; $p < 0.009$, $n = 5$).

We then examined the cell-cycle status of $p53^{-/-}$ $Lin^-Sca\text{-}1^+$ cells using Pyronin Y and Hoechst 33342 staining and observed a reduction of Pyronin Y^{low} cells, indicating the presence of fewer quiescent HSCs ($p < 0.005$, Figure 1A). Using the SP phenotype as a marker for quiescent HSCs in adult bone marrow (Goodell et al., 1996; Arai et al., 2004), we also found a 2-fold decrease in the frequency of $CD34^-$LSK SP cells in the absence of p53 ($p < 0.003$, Figure 1B). Staining of p53 null $CD34^-$LSK cells with the proliferation marker Ki67 also showed enhanced HSC proliferation with fewer quiescent cells present ($p < 0.001$, Figure 1C). Furthermore, to determine the proliferative rate of $p53^{-/-}$ $CD34^-$LSK cells in vivo, we administered BrdU to mice orally for 2 days and isolated $CD34^-$LSK cells from the bone marrow. While ~30% of wild-type $CD34^-$LSK cells incorporated BrdU over this period, ~60% of the $p53^{-/-}$ $CD34^-$LSK cells were BrdU positive (Figure 1D, $p < 0.01$; and Figure S2). These data suggest that p53 promotes HSC quiescence, and in its absence, HSCs more easily enter the cell cycle.

Role of p53 in HSCs of $Mef^{-/-}$ Mice

Given the HSC phenotype of the p53 null mice and the upregulation of p53 in Mef null fibroblasts, we generated $p53^{-/-}Mef^{-/-}$ mice to determine both the frequency and the cell-cycle status of the hematopoietic stem (and progenitor) cells. Loss of MEF alone, p53 alone, or both MEF and p53 increased the frequency of LSK cells (2- to 3-fold) compared to wild-type mice (Figure 2A).

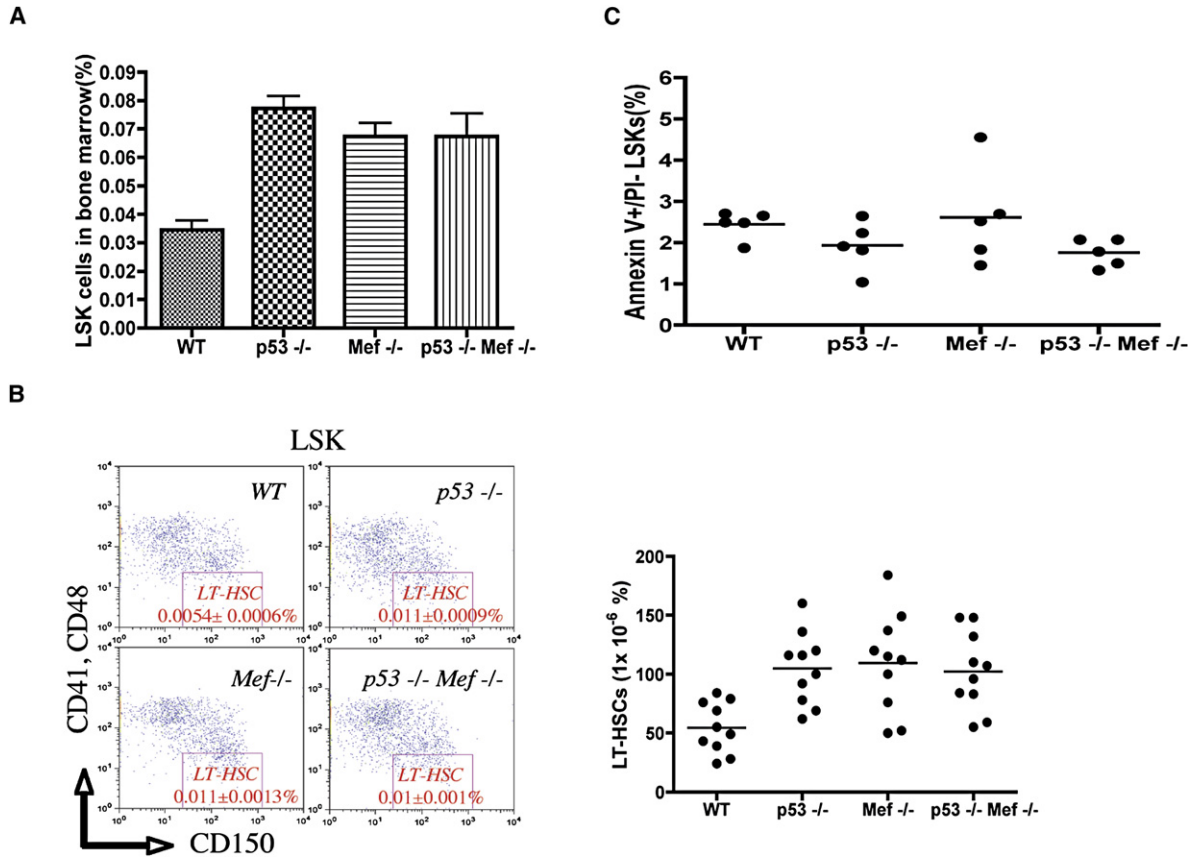

Figure 2. Increased HSC Frequency in $p53^{-/-}Mef^{-/-}$ Mice

(A) Increased LSK cell frequency in $p53^{-/-}Mef^{-/-}$ mice. $Lin^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}$ cells were quantified by flow cytometry. Data shown are the mean percentage (±SD) of LSK cells in the marrow ($p < 0.0002$, $n = 13$).

(B) Increased LT-HSC frequency in the marrow of $p53^{-/-}Mef^{-/-}$ mice. The frequency of LT-HSCs ($CD150^{+}Sca\text{-}1^{+}c\text{-}kit^{+}CD41^{-}CD48^{-}Lin^{-}$) was defined by flow cytometric analysis of SLAM cell surface markers. $p53^{-/-}$, $Mef^{-/-}$, and $p53^{-/-}Mef^{-/-}$ mice all showed a significant increase in the frequency of $CD150^{+}CD41^{-}CD48^{-}$LSK cells, which correspond to LT-HSCs. Data shown are the mean percentage (±SD) of LT-HSCs ($n = 10$).

(C) Bone marrow cells from wild-type, $p53^{-/-}$, $Mef^{-/-}$, and $p53^{-/-}Mef^{-/-}$ mice were stained with stem and progenitor cell surface markers, and apoptosis was assessed using PI and Annexin-V staining. Data shown are mean percentage (±SD) of Annexin-V^{+}/PI^{-} LSK cells ($p = 0.2$ by ANOVA; i.e., statistically not significant, $n = 5$).

This small but consistent increase in HSC frequency was confirmed using the SLAM cell surface makers, as we also observed a consistent 2-fold increase in the frequency of $CD150^{+}CD41^{-}CD48^{-}Sca\text{-}1^{+}c\text{-}kit^{+}Lin^{-}$ cells (LT-HSCs) in the bone marrow of mice lacking MEF, p53, or both MEF and p53 compared to wild-type mice (Figure 2B). The increased HSC numbers could be due to enhanced self-renewal or to decreased apoptosis. We evaluated cell survival by Annexin-V staining and found no significant difference in the number of apoptotic (Annexin-V^{+}/PI^{-}) HSCs in the wild-type and $p53^{-/-}Mef^{-/-}$ mice at steady state (Figure 2C). Thus, the increase in HSCs appears to be due to enhanced self-renewal.

The Increased HSC Self-Renewal Capacity of Mef Null Mice Does Not Depend on p53

To define how loss of p53 (and MEF) affects HSC behavior, we performed in vitro (cobblestone area-forming cell [CAFC], long-term culture-initiating cell [LTC-IC], and serial replating assays) and in vivo (serial bone marrow transplantation and competitive repopulation) functional assays. $p53^{-/-}$ bone marrow cells contain 5-fold more CAFCs compared to wild-type bone marrow cells, and this increased CAFC frequency is also seen in $p53^{-/-}Mef^{-/-}$ bone marrow (Figure 3A). Similar findings were seen in the LTC-IC assay, as $p53^{-/-}$ LSK cells formed 5-fold more colonies than did wild-type LSK cells. However, the $Mef^{-/-}$ and the $p53^{-/-}Mef^{-/-}$ LSK cells formed 10-fold more colonies than the wild-type LSK cells (Figure 3B).

In the serial replating assay, which assesses the preservation of ''stemness'' in the progenitor cell compartment, wild-type BMMCs cannot be replated more than three times. Hematopoietic progenitor cells that lack Mef show increased serial replating in CFU assays, confirming our earlier findings (Lacorazza et al., 2006), and so do the $p53^{-/-}$ and the $p53^{-/-}Mef^{-/-}$ BMMCs (Figure 3C). Thus, loss of p53 showed effects in wild-type HSCs but no detrimental effect on HSC frequency in the Mef null background in any of these in vitro assays.

To assess whether the accumulation of HSCs in $p53^{-/-}Mef^{-/-}$ mice reflects increased self-renewal, we began serial bone

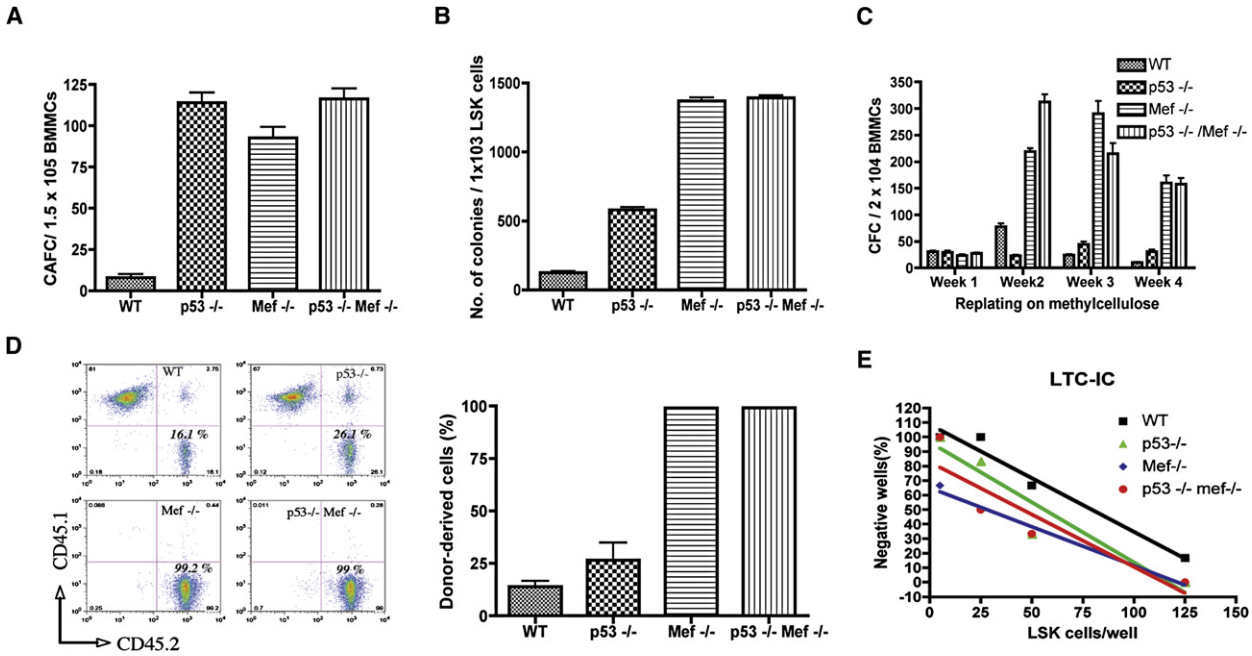

Figure 3. The Increased HSC Self-Renewal Capacity of Mef Null Mice Does Not Depend on p53

(A) The steady-state level of bone marrow cobblestone area-forming cells was evaluated by scoring colonies at week 5. Data shown are mean values (±SD) (n = 3).

(B) LSK cells (1 × 10^3) from wild-type, $p53^{-/-}$, $Mef^{-/-}$, and $p53^{-/-}Mef^{-/-}$ mice were cultured on MS5 stromal cells for 4 weeks and tested for colony formation in the LTC-IC assay. Data shown are the mean number (±SD) of colonies formed (n = 3).

(C) Serial replating studies. Myeloid progenitors were quantified by methycellulose culture using BMMCs from wild-type, $p53^{-/-}$, $Mef^{-/-}$, and $p53^{-/-}Mef^{-/-}$ mice. The methycellulose cultures were serially replated, weekly, for 4 weeks. Mean values (±SD) shown (n = 3).

(D) Lethally irradiated recipient mice (CD45.1) were transplanted with 1 × 10^3 LSK cells from wild-type, $p53^{-/-}$, $Mef^{-/-}$, and $p53^{-/-}Mef^{-/-}$ mice (CD45.2) plus 5 × 10^5 competitor cells (CD45.1) in competitive repopulation assays (described in the Experimental Procedures). The bar graph on the right shows the mean percentage (±SD) of donor-derived (CD45.2) cells in the peripheral blood 16 weeks posttransplantation (n = 4).

(E) The LTC-IC assay was used to enumerate primitive hematopoietic stem cells, using limiting dilutions of LSK cells that were first cultured for 5 weeks on MS5 stroma and then cultured on methylcellulose for the readout. The number of wells that lacked LTC-ICs is graphed versus the number of LSK cells per well.

marrow transplant (sBMT) assays using BMMCs from each genotype (*wild-type*, $p53^{-/-}$, $Mef^{-/-}$, $p53^{-/-}Mef^{-/-}$, C57BL/6, and CD45.2) of mice as donor cells and lethally irradiated mice (CD45.1) as recipients. However, most of the recipient mice transplanted with $p53^{-/-}$ BMMCs died within 4 months post-transplant due to the development of lymphomas, and although recipient mice transplanted with $p53^{-/-}Mef^{-/-}$ BMMCs lived longer, most of them died within 5 months of transplantation. Thus, we could not perform serial bone marrow transplantation. Instead, we performed competitive repopulating experiments, transplanting 1000 LSK cells of each genotype (*wild-type*, $p53^{-/-}$, $Mef^{-/-}$, $p53^{-/-}Mef^{-/-}$, and CD45.2) into lethally irradiated (9.5 Gy) B6.SJL recipient mice (CD45.1) along with a constant, small dose (5 × 10^5) of competitor marrow cells (CD45.1). At 16 weeks posttransplantation, the repopulating abilities of both the $Mef^{-/-}$ and $p53^{-/-}Mef^{-/-}$ cells were many fold higher than the wild-type cells, while loss of p53 had a modest effect on competitive repopulating ability (Figure 3D).

To further assess HSC frequency, we performed long-term culture-initiating cell (LTC-IC) assays, under limiting dilution conditions, a stringent assay that correlates with the in vivo repopulating potential of primitive hematopoietic progenitor cells (Ploemacher et al., 1991). The increased frequencies of primitive HSCs in $Mef^{-/-}$, $p53^{-/-}$, or $p53^{-/-}Mef^{-/-}$ mice were also evident in the LTC-IC assay (Figure 3E). Thus, the enhanced self-renewal potential of $Mef^{-/-}$ HSCs is not dependent on the presence of p53.

p53 Is Essential for Maintaining Hematopoietic Stem Cell Quiescence in Mef Null Mice

We assessed HSC quiescence in the various genetic backgrounds under homeostatic conditions and confirmed the increased quiescence of Mef null HSCs (Figure 4A). We observed a small but consistent decrease in the quiescence of $p53^{-/-}$ HSCs but found that the enhanced quiescence of Mef null HSCs was abrogated by the absence of p53 (analyzing the $p53^{-/-}Mef^{-/-}$ mice). By using the SP phenotype as a marker for quiescent HSCs in adult bone marrow (Arai et al., 2004), we found that p53 loss not only leads to a 2-fold decrease in $CD34^{-}$LSK SP cells in wild-type mice (Figure 1B), but that its absence in Mef null $CD34^{-}$LSK SP cells returns the frequency of these cells in the bone marrow to normal (Figure 4B). Thus, p53 is required for the enhanced stem cell quiescence seen in Mef null mice.

Mef null bone marrow is relatively resistant to radiation (and chemotherapy), an effect that we attributed to the enhanced HSC quiescence (Lacorazza et al., 2006). To assess the effects of both Mef and p53 loss on the response of HSCs to total body irradiation (6.5 Gy), we harvested bone marrow cells 12 hr postirradiation and assayed for apoptosis of LSK cells by

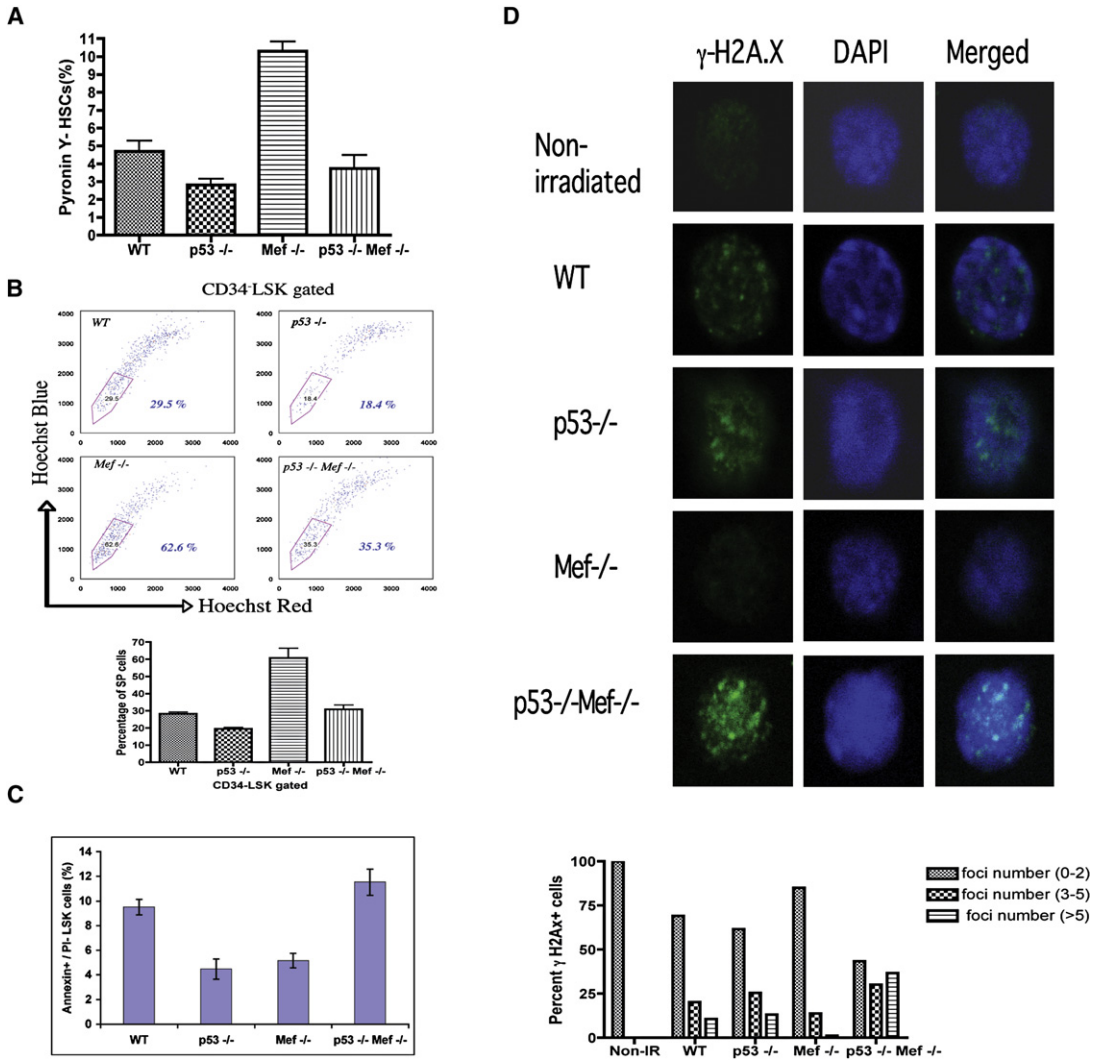

Figure 4. p53 Is Essential for Maintaining Hematopoietic Stem Cell Quiescence in Mef Null Mice

(A) Multicolor flow cytometry was used to determine the percentage of $Lin^{-}Sca\text{-}1^{+}$ cells in the G0 phase of the cell cycle (defined as cells with low Pyronin Y content that contain 2 n DNA [G0/G1]). Data shown are the mean percentage (±SD) (n = 6).

(B) Analysis of SP cells within the $CD34^{-}$LSK cells. $CD34^{-}$LSK cells from wild-type, $p53^{-/-}$, $Mef^{-/-}$, and $p53^{-/-}Mef^{-/-}$ mice were examined for the proportion of SP cells. Data shown are the mean percentage (±SD) of SP cells (n = 3).

(C) Bone marrow cells from wild-type, $p53^{-/-}$, $Mef^{-/-}$, and $p53^{-/-}Mef^{-/-}$ mice obtained 12 hr after a dose of total-body irradiation (TBI, 6.5 Gy) were assessed for apoptosis using PI and Annexin-V staining. Data shown are mean percentage (±SD) of Annexin-V^{+}/PI^{-}LSK cells (n = 3).

(D) γ-H2AX foci generation in HSCs following irradiation. LSK cells from wild-type, $p53^{-/-}$, $Mef^{-/-}$, and $p53^{-/-}Mef^{-/-}$ mice were immunostained for γ-H2AX 3 hr after irradiation (200 rads) (and DAPI stained to identify the nuclei). The bar graph at the bottom shows the percentage of LSK cells that show 0–2, 3–5, or >5 foci (200 cells are counted in each genotype).

Annexin-V staining. As expected, LSK cells from both the $p53^{-/-}$ mice and the $Mef^{-/-}$ mice showed decreased apoptosis after radiation. However, LSK cells from the $p53^{-/-}Mef^{-/-}$ mice exhibited normal radiation-induced apoptosis (Figure 4C), implying that abrogating the enhanced HSC quiescence in the $Mef^{-/-}$ mice is more important in defining radio-sensitivity than is abrogating p53-dependent apoptosis.

As phosphorylation of histone H2AX (γ-H2AX) is an indicator of DNA damage (Rossi et al., 2007), we irradiated wild-type, $p53^{-/-}$, $Mef^{-/-}$, and $p53^{-/-}Mef^{-/-}$ mice (2 Gy) and quantified the number of γ-H2AX foci in individual HSCs using immunostaining. We found that HSCs from $Mef^{-/-}$ mice were largely devoid of γ-H2AX foci (15% cells stain positive for 3–5 foci of γ-H2AX and none for >5 foci), while more than 30% of the HSCs from wild-type, $p53^{-/-}$, and $p53^{-/-}Mef^{-/-}$ mice stained positively for γ-H2AX (Figure 4D). These data further indicate that the relative radioresistance of HSCs from $Mef^{-/-}$ mice, like the enhanced quiescence, requires the presence of p53.

HSC Quiescence in Mef Null Mice Does Not Depend on p21

p21 is a major target gene of p53, and in the absence of p21, HSC numbers and proliferation are reported to be increased, leading to stem cell exhaustion following serial bone marrow

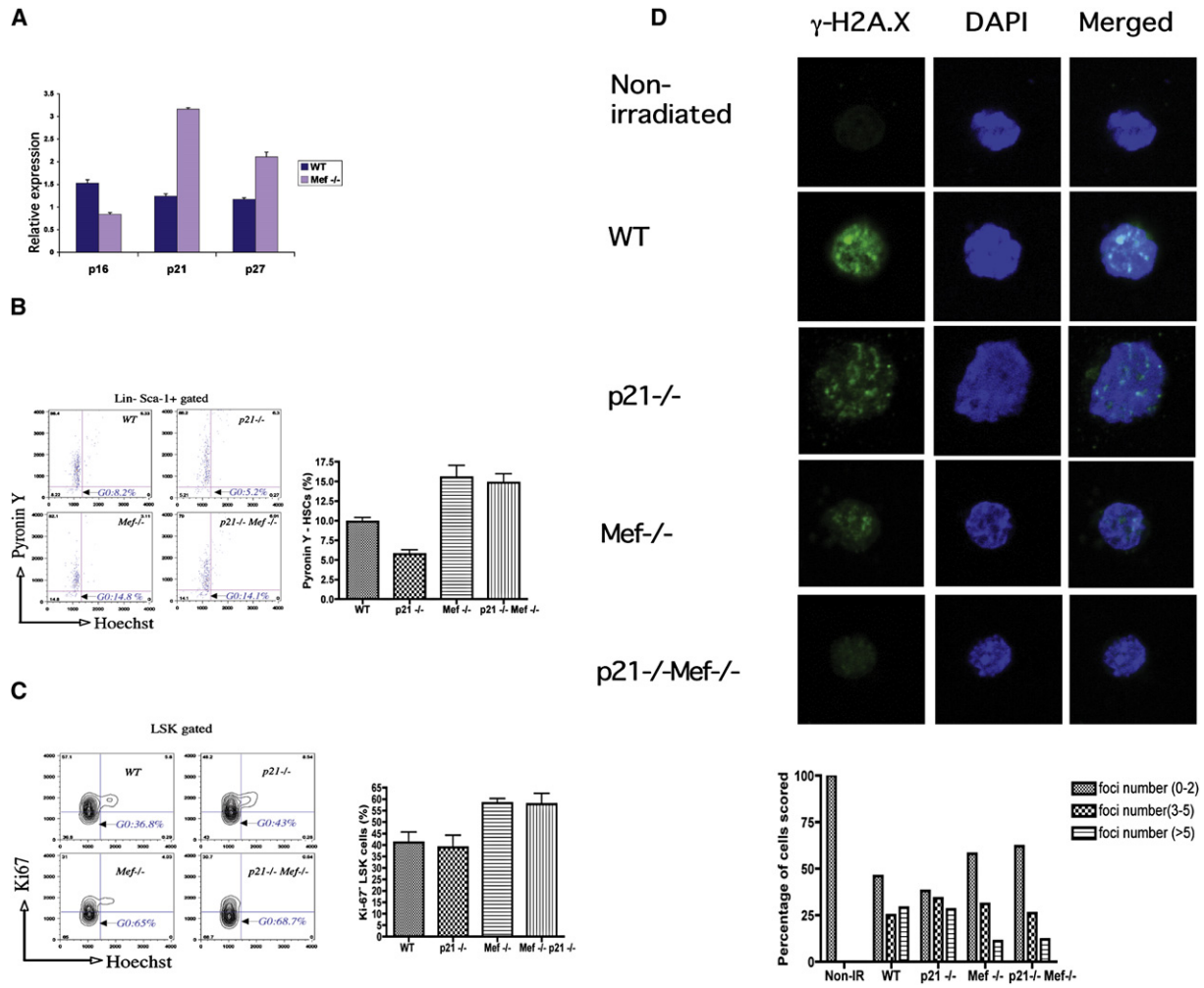

Figure 5. Enhanced HSC Quiescence in Mef Null Mice Does Not Depend on p21

(A) Increased p21 and p27 expression in $Mef^{-/-}$ LSK cells. The relative mRNA expression level of p21, p27, and p16 in LSK cells from wild-type and $Mef^{-/-}$ mice were evaluated by qPCR and normalized to HPRT expression. Data shown are the mean ratio (±SD) of transcript levels over HPRT (n = 2).

(B) Multicolor flow cytometry was used to determine the percentage of hematopoietic stem cells in the G0 phase of the cell cycle. $Lin^{-}Sca\text{-}1^{+}$ cells in G0 phase are defined as cells with low Pyronin Y content that contain 2n DNA (G0/G1). The bar graph on the right shows the mean percentage (±SD) (n = 5).

(C) Cell-cycle analysis of LSK cells from wild-type, $p21^{-/-}$, $Mef^{-/-}$, and $p21^{-/-}Mef^{-/-}$ mice. Cells were stained with Hoechst 33342 and Ki67 and analyzed by FACS. Data shown are the mean percentage (±SD) (n = 6).

(D) γ-H2AX foci generation in HSCs following irradiation. LSKs from wild-type, $p21^{-/-}$, $Mef^{-/-}$, and $p21^{-/-}Mef^{-/-}$ mice were immunostained for γ-H2AX 3 hr after irradiation (200 rads). DAPI staining was used to identify the nuclei. The bar graph at the bottom shows the percentage of LSK cells that show 0–2, 3–5, or >5 foci (200 cells are counted for each genotype).

transplantation (Cheng et al., 2000). As Mef null LSK cells show increased quiescence, we first examined whether p21 expression is upregulated in $Mef^{-/-}$ HSCs and could account for their enhanced quiescence (and for the effect that loss of p53 has on HSC quiescence). Real-time PCR analysis determined that the level of p21 expression is several-fold higher in $Mef^{-/-}$ HSCs than in wild-type HSCs; p27 is modestly upregulated and p16 is downregulated (Figure 5A).

We then generated $p21^{-/-}Mef^{-/-}$ mice, which are viable, born at normal Mendelian frequency, and appear grossly normal. Based on Pyronin Y and Hoechst 33342 staining, we found no effect of p21 loss on the enhanced HSC quiescence generated by the lack of Mef (Figure 5B). Furthermore, the aberrantly high proportion of noncycling ($Ki67^{-}$) LSK cells seen in the $Mef^{-/-}$ mice was also seen in the $p21^{-/-}Mef^{-/-}$ mice (Figure 5C and Figure S3). Thus, the increased quiescence of Mef null HSCs is not dependent on p21.

To further address whether the relative radioresistance of the Mef null HSCs reflected their quiescence status, we irradiated wild-type, $p21^{-/-}$, $Mef^{-/-}$, and $p21^{-/-}Mef^{-/-}$ mice (2 Gy) and quantified the number of γ-H2AX foci in individual HSCs. HSCs from both $Mef^{-/-}$ and $p21^{-/-}Mef^{-/-}$ mice had many fewer γ-H2AX foci than HSCs from either wild-type or $p21^{-/-}$ mice (Figure 5D). Thus, the relative radioresistance of HSCs in $Mef^{-/-}$ mice, like the enhanced quiescence, does not require the presence of p21.

Gfi-1 and *Necdin* Are Direct Transcriptional Targets of p53 and Mediate Its Effects in HSCs

The quiescent state of Mef null HSCs is p53 dependent but p21 independent, which implies that other p53 target genes are involved in maintaining stem cell quiescence. To identify such p53 targets, we performed transcript profiling (using microarray studies and quantitative real-time PCR analysis) to compare gene expression in LSK cells isolated from wild-type, $p53^{-/-}$, and $p53^{-/-}Mef^{-/-}$ mice. We also utilized Ingenuity Pathways Analysis software to group potential p53 target genes into specific pathways that may be important in HSC behavior. We identified several putative p53 target genes (that are differentially expressed in both $p53^{-/-}$ and $p53^{-/-}Mef^{-/-}$ cells compared to wild-type cells) and several signaling pathways that appear to be altered in the absence of p53 (Figure 6A and Figure S6). Two genes drew our initial attention: *Gfi-1* and *Necdin*. Gfi-1 (growth factor independent-1) is a zinc finger containing a transcriptional repressor that has been shown to restrict HSC proliferation and preserve HSC functional integrity (Hock et al., 2004; Zeng et al., 2004). Necdin is another growth suppressor. It acts as a negative cell-cycle regulator in postmitotic neurons and contributes to their permanent mitotic arrest (Hu et al., 2003; Taniura et al., 1999, 2005; Yoshikawa, 2000); necdin is known to be highly expressed in LT-HSCs (Forsberg et al., 2005).

We first confirmed the marked increase of *Gfi-1* and *Necdin* mRNA in $Mef^{-/-}$ LSK cells by real-time PCR analysis (shown in Figure 6B). To determine whether *Gfi-1* and *Necdin* are direct targets of p53, we scrutinized the DNA sequence of the mouse and human *Gfi-1* and *Necdin* genes and found two conserved potential p53 binding sites (p53 RE1 and p53 RE2) in the *Gfi-1* promoter and one conserved p53 binding site in the *Necdin* promoter. To assess whether any of these sites are essential for p53-mediated activation, each site was individually mutated in a luciferase reporter plasmid: p53 strongly transactivated the *Necdin* promoter, and point mutations in the consensus p53 binding site abolished p53-mediated promoter activation (Figure 6C). p53 also transactivated the *Gfi-1* promoter 5-fold. Mutations in the p53 RE1, but not the p53 RE2, abolished p53-mediated Gfi-1 promoter activation, implying that only the p53 RE1 is required for the p53 responsiveness of the Gfi-1 promoter.

Next, to determine whether endogenous p53 is bound to the *Gfi-1* and *Necdin* promoters in vivo, we performed chromatin immunoprecipitation (ChiP) assays and observed binding of p53 to both the *Gfi-1* and *Necdin* promoters in Lin^- bone marrow cells (Figure 6D). Thus, p53 directly binds the *Gfi-1* and *Necdin* promoters and activates their expression.

The Role of Necdin in Hematopoiesis

To examine whether Gfi-1 and Necdin are responsible for the enhanced quiescence of Mef null HSCs, we acutely lowered their expression in $Lin^-Sca\text{-}1^+$ cells, using siRNA. First, we reduced the level of both Gfi-1 and Necdin mRNA and found significant reduction in the quiescence of the $Mef^{-/-}$ HSCs cells (Figure 6E), indicating that these p53 target genes are functionally important in HSCs.

While Gfi-1 is a known regulator of HSC proliferation (Hock et al., 2004), the function of Necdin in regulating quiescence in normal HSCs has not been described. To further investigate its role in HSC biology, we measured Necdin mRNA levels in LSK cells (and in a variety of committed myeloid progenitor cells); Only the LSK cells had significant Necdin expression (Figure S7). Then, we reduced Necdin expression in wild-type $Lin^-Sca\text{-}1^+$ cells using siRNA and also overexpressed Necdin in $Lin^-Sca\text{-}1^+$ cells using lentiviral gene transfer. HSC quiescence decreased when Necdin was knocked down and increased when we overexpressed Necdin (based on the percentage of $Lin^-Sca\text{-}1^+$ cells in the G0 phase of the cell cycle, Figures 7A and 7B). Thus, Necdin appears to be a true regulator of HSC quiescence.

To examine if Necdin also regulates HSC self-renewal, we knocked-down Necdin expression in mouse bone marrow cells using lentivirally expressed shRNA and performed in vitro serial replating assays. The downregulation of Necdin in wild-type bone marrow cells significantly increased colony numbers at week 1 and, to a greater extent, in weeks 2 and 3 (Figure 7C). Thus, like p53, Necdin also regulates the maintenance of HSC self-renewal capability, recapitulating another effect of p53 on HSC biology.

DISCUSSION

p53 plays critical roles in triggering senescence, apoptosis and cell cycle arrest in virtually all cell types (Vousden and Lane, 2007). We have investigated its role in hematopoietic stem/progenitor cells (i.e., LSK cells) and have found effects on HSC quiescence and self-renewal. Using a variety of in vivo and in vitro assays, we have shown that HSC quiescence is impaired in the absence of p53 and that p53 function is essential for the enhanced stem cell quiescence seen in Mef null mice.

p53-dependent downstream responses have been shown to be mediated by specific target genes, such as p21, BTG2, BAX, and PUMA (Zhao et al., 2000; Boiko et al., 2006; Meletis et al., 2006). Comparative transcript profiling has allowed us to identify a variety of p53 target genes in HSCs; some of these genes are inversely regulated by MEF, suggesting that p53 function is enhanced in the absence of MEF. To our surprise, the increased stem cell quiescence observed in Mef null mice is not dependent on p21, despite its elevated level, which prompted us to look for other p53 target genes that could play this role.

To identify such p53 targets, we compared gene expression in LSK cells isolated from wild-type, $p53^{-/-}$, and $p53^{-/-}Mef^{-/-}$ mice and identified several potential regulators of HSC self-renewal and proliferation that were differentially expressed between wild-type and $p53^{-/-}$ HSCs (Figure 6A). The expression of two of these genes (CCNG1 and SERPINE2) was previously reported to be controlled by p53, validating the approach. However, the majority of differentially expressed genes have not been implicated as p53 targets. Some of the differentially expressed genes fit into signaling pathways that may affect HSC maintenance, such as Wnt, p38 MAPK signaling, PTEN signaling, and cytokine signaling (Chambers et al., 2007). For example, SOCS3 downregulates cytokine signaling, and the Wnt receptor FZD7 has been implicated in the self-renewal of human embryonic stem cells (Melchior et al., 2008); both are upregulated in $p53^{-/-}$ and $p53^{-/-}Mef^{-/-}$ HSCs and could play a role in the regulation of HSC behavior (Figure 6A). Caspase-3, a downstream target of the PTEN/Akt pathway recently implicated in regulating the responsiveness of HSCs to exogenous signals (Janzen et al., 2008), is also upregulated in both $p53^{-/-}$ and $p53^{-/-}Mef^{-/-}$ HSCs. Thus, caspase-3 upregulation may also contribute to the enhanced

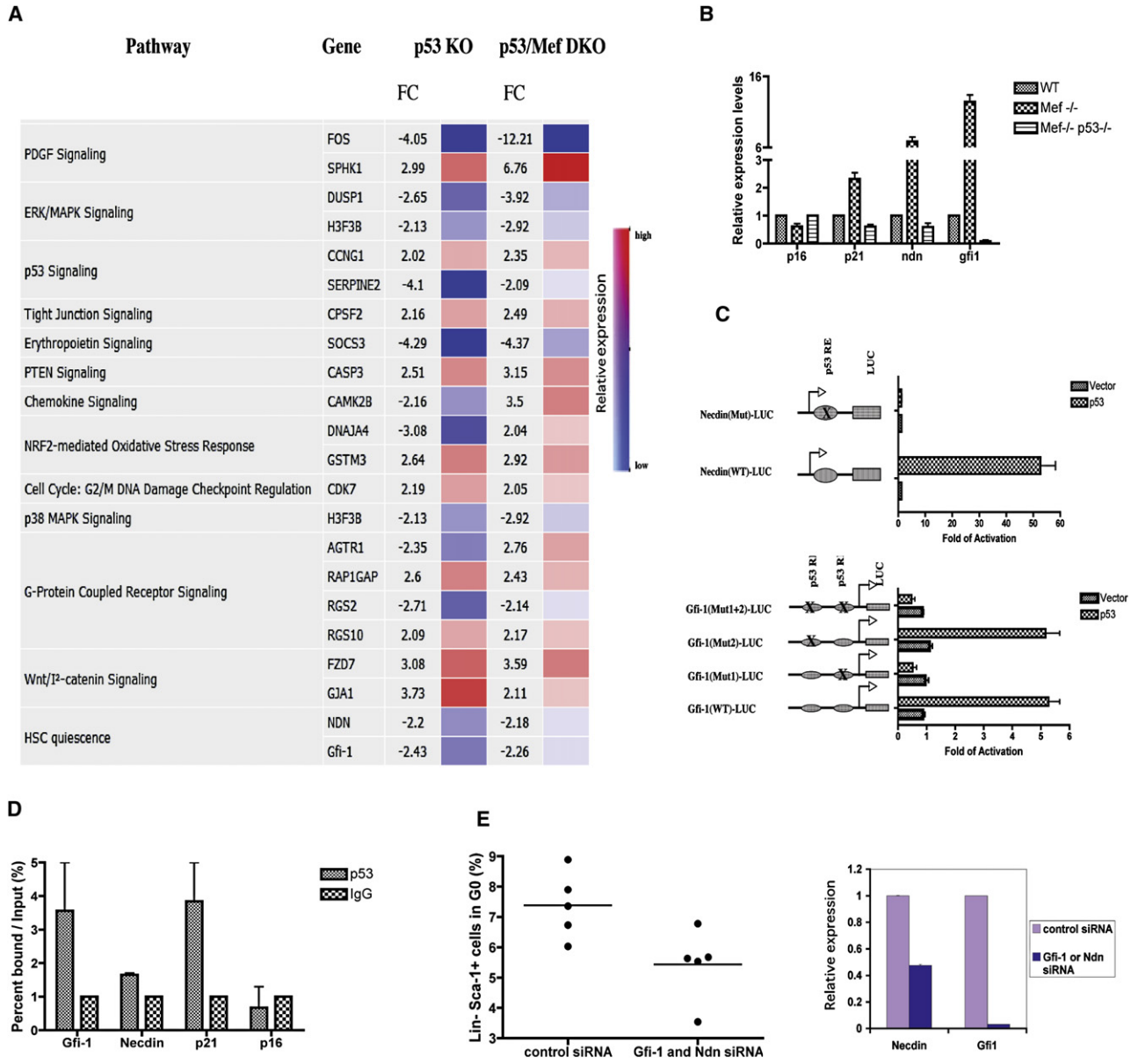

Figure 6. *Gfi-1* and *Necdin* Are Direct Transcriptional Targets and Functional Mediators of p53 in HSCs

(A) Transcript profiling of LSK cells isolated from wild-type, $p53^{-/-}$, $p53^{-/-}Mef^{-/-}$ mice were analyzed by Affymetrix oligonucleotide array. Genes that are differentially expressed in both $p53^{-/-}$ and $p53^{-/-}Mef^{-/-}$ HSCs compared to wild-type cells are shown. We utilized Ingenuity Pathways Analysis (Ingenuity Systems) to group genes into specific canonical pathways. FC stands for fold change.

(B) Increased Gfi-1 and Necdin expression in $Mef^{-/-}$ LSK cells. The relative mRNA expression level of p16, p21, Gfi-1, and Necdin in LSK cells from wild-type, $Mef^{-/-}$ and, $p53^{-/-}Mef^{-/-}$ mice were evaluated by qPCR and normalized to HPRT expression. Data shown are the mean ratio (±SD) of transcript levels relative to HPRT (n = 2).

(C) p53 transactivates both the *Necdin* and *Gfi-1* promoters. HeLa cells were transfected with *Necdin* promoter- or *Gfi-1* promoter-driven luciferase reporter plasmids containing either wild-type p53 binding sites or mutant p53 binding sites. Luciferase activity was assayed 24 hr after transfection. Values are means (±SD) (n = 3).

(D) p53 binds to the Gfi-1 and Ndn promoters in vivo. Chromatin bound DNA from Lin^- bone marrow cells was immunoprecipitated with a p53-specific antibody or with normal mouse IgG. qPCR amplification was performed on corresponding templates using primers for the *Gfi-1*, *Necdin*, or *p21* genes.

(E) Downregulation of Gfi-1 and Necdin expression in $Mef^{-/-}$ cells decreases HSC quiescence. $Mef^{-/-}$ $Lin^-Sca\text{-}1^+$ cells were nucleofected with control, Gfi-1 or Necdin siRNAs. Twenty-four hours postnucleofection, cells were stained with Pyronin Y and Hoechst 33342 and analyzed by FACS. Values are means (±SD) ($p < 0.004$, n = 5). The effectiveness of the knockdown for each siRNA (versus control siRNA) is shown on the right.

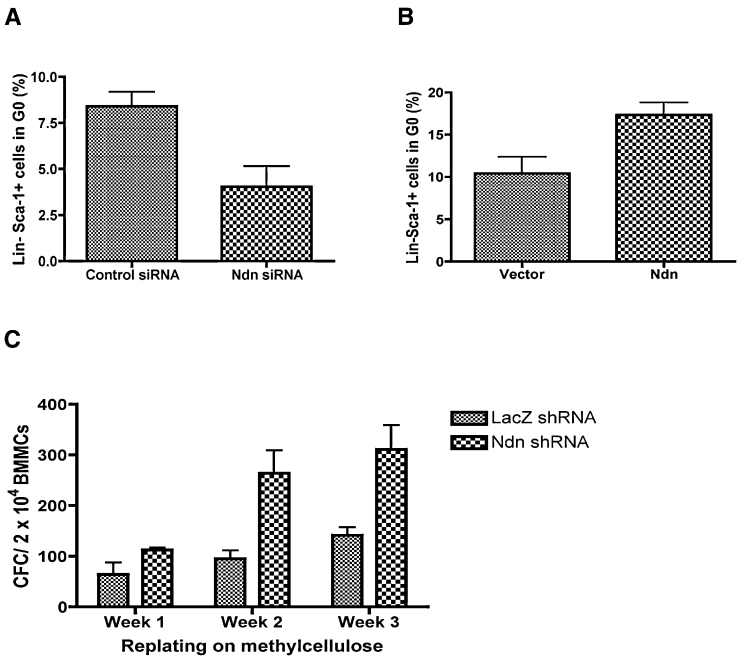

Figure 7. Necdin Functions as a Rheostat to Regulate HSC Quiescence and Maintenance

(A) Effect of downregulating Necdin expression on HSC quiescence. Wild-type Lin$^-$ cells were nucleofected with control or Necdin siRNAs. Twenty-four hours postnucleofection, the cells were stained with Pyronin Y and Hoechst 33342 and analyzed by FACS. Values are means (±SD) ($p < 0.04$, $n = 2$).

(B) Effect of overexpressing Necdin on HSC quiescence. Wild-type Lin$^-$ cells were infected with control or Necdin-expressing lentiviruses. Forty-eight hours postinfection, the cells were stained with Pyronin Y and Hoechst 33342 and analyzed by FACS. Values shown are the means (±SD) ($p < 0.02$, $n = 3$).

(C) Effects of downregulating Necdin expression on serial replating of hematopoietic stem/progenitor cells in methycellulose assays. Values shown are means (±SD) ($n = 3$).

self-renewal of *p53*$^{-/-}$ HSCs. While changes in the levels of these genes may alter HSC behavior, we expect that no one p53 target will be absolutely essential for its effect on quiescence. While we have identified genes that are differentially expressed between *p53*$^{-/-}$ and *p53*$^{-/-}$*Mef*$^{-/-}$ HSCs, these genes are likely to be regulated by MEF/ELF4, and they will not be further discussed.

There is accumulating evidence for the role of tumor suppressor genes and oncogenes in stem cell maintenance and cell cycle regulation. While our transcript profiling of hematopoietic stem cells lacking p53 provides an overview of the pathways that may be important for HSC quiescence and self-renewal, pathways not yet implicated in regulating HSC behavior may still play an important role. As not all hematopoietic proteins are identified by the pathway analysis software, we have focused primarily on Necdin, a negative regulator of the cell cycle (Hu et al., 2003; Taniura et al., 1999, 2005; Yoshikawa, 2000) that is highly expressed in HSCs (Figure S7 and Forsberg et al., 2005). We find that the enhanced quiescence exhibited in Mef null HSCs is, at least in part, mediated by Necdin. Furthermore, downregulating Necdin diminishes HSC quiescence, and upregulating Necdin increases HSC quiescence, identifying its role as a rheostat controlling HSC quiescence (Figures 7A and 7B). Necdin and p53 have been shown to inhibit cell growth in an additive manner (Taniura et al., 1999), suggesting the presence of a positive feedback loop that may control the quiescent state (Figure S6). While disruption of the mouse necdin gene results in early postnatal lethality with variable penetrance in C57BL/6 background (Gérard et al., 1999; Muscatelli et al., 2000), no postnatal lethality is seen in the ICR strain background (Kuwako et al., 2005). Analysis of HSC behavior in necdin-deficient mice will further define the role of necdin in fetal and/or adult hematopoiesis. Gfi-1 is another p53 target identified in our ChiP and reporter gene assays. The *Gfi-1* locus is among the most frequent sites for retroviral integration contributing to the development of lymphoid tumors in mice (Zhu et al., 2002); its role in HSCs is well documented (Hock et al., 2004; Zeng et al., 2004). However, we have identified Gfi-1 as a direct transcriptional target of p53 in HSCs and confirm its importance in regulating cell-cycle progression in these cells.

Using γ-H2AX foci formation as an indicator of DNA damage (Rossi et al., 2007), we found that LT-HSCs (CD34$^-$LSKs) formed less γ-H2AX foci than ST-HSCs (CD34$^+$LSKs) (Figure S4). LT-HSCs are more quiescent than ST-HSCs (Passegué et al., 2005); thus, γ-H2AX foci formation in HSCs seems to correlate with the quiescent status of the cell. Consistent with the enhanced quiescence of Mef$^{-/-}$ HSCs, we found that only 15% HSCs from *Mef*$^{-/-}$ mice formed γ-H2AX foci after irradiation, compared to 56% of the HSCs from *p53*$^{-/-}$*Mef*$^{-/-}$ mice, which are less quiescent. Mef null LSKs undergo less apoptosis than wild-type LSKs after irradiation, whereas *Mef*$^{-/-}$*p53*$^{-/-}$ LSKs show normal radiosensitivity. Thus, HSC quiescence tracks with radioresistance in both the Mef null and Mef/p53 double null mice. We would predict that because the enhanced HSC quiescence is maintained in the *p21*$^{-/-}$*Mef*$^{-/-}$ mice, the enhanced recovery from myelosuppressive treatments seen in *Mef*$^{-/-}$ mice should also be preserved. Indeed, we find that *p21*$^{-/-}$*Mef*$^{-/-}$ mice have less leukopenia following a single dose of 5-flurouracil (5-FU) than wild-type mice and more rapid recovery (Figure S5), a pattern identical to that seen for the *Mef*$^{-/-}$ mice. Thus, p21 is not essential for the relative quiescence or the chemoresistance seen in Mef null mice. These findings provide additional proof that the resistance of HSCs to irradiation, or chemotherapy agents, depends strongly on their quiescent properties.

HSCs are sensitive to reactive oxygen species (ROS), as elevated ROS levels can limit the life span of HSCs in vivo (Ito et al., 2006). Loss of p53 function in normal cells increases intracellular levels of ROS, which leads to increased DNA damage and an increased mutation rate (Sablina et al., 2005). The relative quiescence of LT-HSCs may protect these cells from DNA damage caused by ROS, chemotherapy drugs, or irradiation. Avoiding inheritable damage would be particularly important for stem cells, and a cytoprotective function of p53 in the hematopoietic compartment, mediated by its effects on HSC quiescence, would help maintain HSC integrity.

We have established a role of p53 in steady-state hematopoiesis which is more profoundly seen in the absence of Mef. Our report adds to the evidence that p53 negatively regulates HSC

self-renewal (TeKippe et al., 2003; Chen et al., 2008; Akala et al., 2008), as we too find that $p53^{-/-}$ mice have more immunophenotypic HSCs. But we also found that $p53^{-/-}$ mice have more functional HSCs as measured by a variety of in vitro and in vivo assays. Additionally, the enhanced self-renewal of $Mef^{-/-}$ HSCs does not depend on the presence of p53 (Figure 3), suggesting that other self-renewal regulators mediate this effect. As p53 has been shown to negatively regulate proliferation and self-renewal of neural stem cells (Meletis et al., 2006), our work provides additional proof that neural stem cells and hematopoietic stem cells utilize common pathways to maintain their stem cell compartment (Molofsky et al., 2003; Park et al., 2003; Groszer et al., 2006; Yilmaz et al., 2006; Zhang et al., 2006).

Mutations within the stem cell can ultimately lead to the generation of leukemic stem cells, which often retain many characteristics of normal hematopoietic stem cells such as a hierarchical developmental pattern, a mostly quiescent cell-cycle profile, and an immunophenotype very similar to HSCs (Jordan and Guzman, 2004; Jin et al., 2006; Komarova and Wodarz, 2007). The tumor suppressor gene p53 is the most mutated gene in human cancer, and further study is needed to determine whether gain-of-function p53 mutations that contribute to chemotherapy and radiation therapy resistance still promote the quiescence of hematopoietic stem cells. Our findings have important implications for HSCs homeostasis during steady-state hematopoiesis and for developing therapeutic strategies that could eliminate the largely quiescent cancer stem cell.

EXPERIMENTAL PROCEDURES

Mice

The generation of Mef-deficient mice was described previously (Lacorazza et al., 2006). $p21^{-/-}$ mice (129/sv) were kindly provided by Tyler Jacks and $p53^{+/-}$ mice (C57BL/6, CD45.2) by Carlos Cordon-Cardo. $p21^{-/-}Mef^{-/-}$ mice were generated by interbreeding $p21^{-/-}$ female with $Mef^{-/-}$ male mice, and $p53^{-/-}Mef^{-/-}$ (C57BL/6, CD45.2) mice were generated by interbreeding $p53^{+/-}$ female with $Mef^{-/-}$ male mice. Wild-type C57BL/6 (CD45.2) and B6.SJL (CD45.1) mice were purchased from Jackson Laboratories. All mice were maintained in the MSKCC Animal Facility according to IACUC-approved protocols and kept in Thorensten units with filtered germ-free air.

Flow Cytometry

Murine hematopoietic stem/progenitor cells (LSK cells) were identified and evaluated by flow cytometry using a single cell suspension of bone marrow mononuclear cells (BMMCs). BMMCs were obtained from both tibias and femurs by flushing cells out of the bone using a syringe and DMEM + 10% FBS. Cells were first stained with a lineage (Lin) cocktail of antibodies from BD Biosciences (biotinylated anti-mouse antibodies directed against CD3e, CD11b, CD45R/B220, Gr-1, and Ter119), as well as Sca-1 PE and c-kit APC (PharMingen) and a streptavidin Cychrome conjugate (PharMingen) for analysis using a FACScan cytometer (Becton Dickinson). c-kit APC, Sca-1 PE-Cy7, Flt3 PE, CD34 FITC, and streptavidin APC-Cy7 were used for analysis using a Moflow cytometer (Cytomation). FITC-CD41, FITC-CD48, and FITC-CD34 were purchased from eBioscience, and PE-CD150 was purchased from Biolegend for SLAM marker analysis. All other antibodies were from BD Biosciences. CMP, GMP, and MEP populations were analyzed and sorted with a Moflow instrument (Cytomation) (Akashi et al., 2000). Nuclear staining of Ki67 was done using an FITC-anti-human Ki67 antibody (BD PharMingen) and fixation and permeabilization solutions from BD Biosciences.

Stem and Progenitor Cell Assays

To perform the CAFC assay, we seeded 1.5×10^5 bone marrow cells onto MS5 stroma cells and cultured the cells in αMEM containing 12.5% FBS, 12.5% horse serum, 1 μM hydrocortisone, and 1 mM glutamine. The media was semi-replenished weekly, and the "cobblestone" colonies were scored at week 5 and expressed as the number of CAFC per 1.5×10^5 BMMCs. CAFC frequency was determined using L-Calc software (StemCell Technologies).

For the long-term culture initiating cell (LTC-IC) assay, 1×10^3 LSK cells were cultured on MS5 stromal cells. After 4 weeks of weekly semi-replenishment of the media, cells were harvested and plated on methylcellulose media (MethoCult GF M3434, StemCell Technologies). Clonogenic progenitors were determined after 10 days using 2×10^4 cells per well (6-well plate). Colonies were scored and expressed as number of the CFUs per 1×10^3 LSK cells.

For the limiting dilution LTC-IC assays, serial 5-fold dilutions were done using initially 125 LSKs/well; 6 replicates of each genotype (WT, $p53^{-/-}$, $Mef^{-/-}$, and $p53^{-/-}Mef^{-/-}$) were performed. After 5 weeks of weekly semireplenishment of the media, cells were trypsinized and plated on methylcellulose media (MethoCult GF M3434, StemCell Technologies) and cultured for 10 days before the percentage of negative wells per dilution was scored. Frequencies were calculated using Poisson statistics (L-cal program from StemCell Technologies).

Bone Marrow Transplantation

In the competitive repopulation study, we injected 1000 LSK cells from wild-type, $Mef^{-/-}$, $p53^{-/-}$, and $Mef^{-/-}p53^{-/-}$ mice (CD45.2) together with 5×10^5 competitor cells (CD45.1) into lethally irradiated B6.SJL mice (CD45.1). After 16 weeks, peripheral blood was obtained by retro-orbital eye bleeding, the RBCs were lysed, and the PBMCs were stained with anti-CD45.2 FITC and anti-CD45.1 PE and analyzed by flow cytometry.

γ-H2AX Immunostaining

γ-H2AX was revealed by using the SCIPhos (single-cell imaging of phosphorylation) assay (Rossi et al., 2007). In brief, HSCs ($CD34^-$LSK and $CD34^+$LSK) were sorted into droplets of PBS on poly(l-lysine)-coated slides, then fixed, permeabilized, and stained with phospho-specific (Ser 139) histone H2AX antibody (Biolegend). Fluorescence images were obtained by using a confocal laser-scanning microscope (Olympus, model FV1000). We quantified γ-H2Ax foci number in individual stem cells and counted 200 cells in each group.

Gene Expression and Pathways Analysis

Gene expression assays were performed as described previously (Jankovic et al., 2007), using RNAs isolated from wild-type, $p53^{-/-}$, and $p53^{-/-}Mef^{-/-}$ LSK cells in both oligonucleotide arrays (Affymetrix) and qPCR. The DNA sequence of all PCR primer pairs used is available upon request. Raw data will be available for download from Gene Expression Omnibus (http://ncbi.nlm.nih.gov/geo/, accession number GSE13448). The Affymetrix data were analyzed using the Ingenuity Pathways Analysis program (Ingenuity Systems, www.ingenuity.com); to identify the pathways that met the < or > 2-fold change cutoff and were associated with a canonical pathway in the Ingenuity Pathways Knowledge base were considered for the analysis. The significance of the association between the data set and the identified canonical pathway was measured in 2 ways: (1) A ratio of the number of genes from the data set that map to the pathway divided by the total number of genes from the data set that map to the canonical pathway and (2) Fischer's exact test, to calculate a p value determining the probability that the association between the genes in the data set and the canonical pathway is explained by chance alone.

Electroporation of siRNAs into $Lin^-Sca\text{-}1^+$ cells

1×10^6 lineage negative mouse bone marrow cells were nucleofected using the mouse Macrophage Nucleofector Kit (Amaxa) with program Y-01 and 100 pmol siRNA. Cells were then cultured in serum-free medium (X vivo-15) in the presence of cytokines (SCF, 100 ng/ml; TPO, 100 ng/ml; Flt 3 Ligand, 100 ng/ml). Twenty-four hours postnucleofection, the cells were analyzed by flow cytometry.

Statistics

Statistical significance was assayed by Student's t test and one-way Anova; $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.005$; ns, not significant.

SUPPLEMENTAL DATA

The Supplemental Data include seven figures, Supplemental Experimental Procedures, and Supplemental References and can be found with this

article online at http://www.cell.com/cell-stem-cell/supplemental/S1934-5909(08)00577-8.

ACKNOWLEDGMENTS

The authors would like to thank the staff of the Flow Cytometry, Mouse Genotyping, Molecular Cytology, and Genomics Core Facilities in MSKCC. We are also grateful to Dr. Keisuke Ito for his suggestions on conducting SP analysis and to Dr. John Petrini for his critical review of our manuscript. We thank Kirin for providing cytokines. We also would like to thank the Wally Yonamine Fund for Leukemia Research. This work was funded by NIH RO1 grant (DK52208 to S.D.N.) and an LLS SCOR grant (S.D.N.).

Received: March 27, 2008
Revised: September 19, 2008
Accepted: November 10, 2008
Published: January 8, 2009

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